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Review

Molecular Features of Calcific Aortic Stenosis in Female and Male Patients

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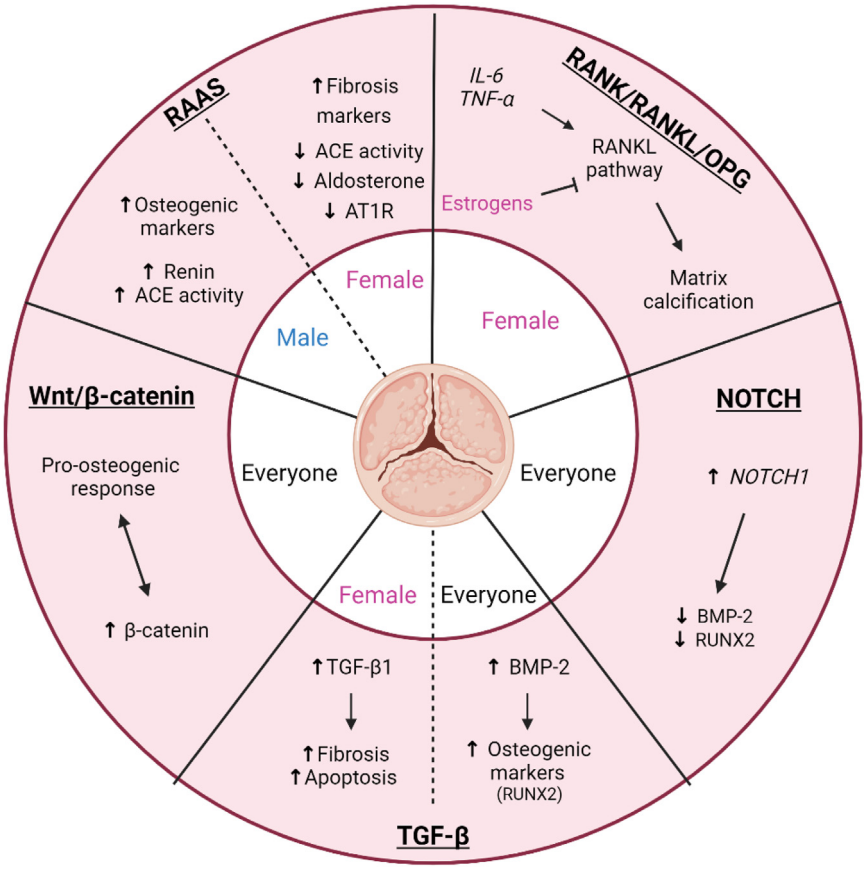

ABSTRACT

Over the past 15 years, sex-related differences in aortic valve (AV) stenosis (AS) have been highlighted, affecting various aspects of AS, such as the pathophysiology, AV lesions, left ventricle remodelling, and outcomes. Female patients were found to present a more profibrotic pattern of leaflet remodelling and/or thickening, whereas male patients have a preponderance of calcification within stenosed leaflets. The understanding of these sex differences is still limited, owing to the underrepresentation of female patients in many basic and clinical

RÉSUMÉ

Au cours des 15 dernières années, des différences liées au sexe dans la sténose (SA) de la valve aortique (VA) ont été mises en évidence, affectant divers aspects de la SA, tels que la pathophysiologie, les lésions de la VA, le remodelage du ventricule gauche et les pronostics associés. Il a été constaté que les patientes présentaient un patron plus profibrotique du remodelage des feuillets ou de leur épaississement, tandis que les patients de sexe masculin voyaient une prépondérance de la calcification au sein des feuillets sténosés. La

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research studies and trials. A better understanding of sex differences in the pathophysiology of AS may highlight new therapeutic targets that potentially could be sex-specific. This review aims to summarize sex-related differences in AS, as discovered from basic research experiments, covering aspects of the disease ranging from leaflet composition to signalling pathways, sex hormones, genetics and/or transcriptomics, and potential sex-adapted medical treatments.

compréhension de ces différences entre les sexes est encore limitée, en raison de la sous-représentation des femmes dans de nombreuses études et essais de recherche fondamentale et clinique. Une meilleure compréhension des différences entre les sexes dans la physiopathologie de la SA pourrait mettre en évidence de nouvelles cibles thérapeutiques qui pourraient être spécifiques au sexe. Cette revue vise à résumer les différences liées au sexe dans la SA, telles qu'elles ont été découvertes par des expériences de recherche fondamentale, couvrant des aspects de la maladie allant de la composition du feuillet aux voies de signalisation, aux hormones sexuelles, à la génétique ou à la transcriptomique, ainsi qu'aux traitements médicaux potentiels adaptés en fonction du sexe.

Calcified aortic valve (AV) stenosis (AS) is the most common valvular heart disease in high-income countries. AS occurs in about 5% of the population aged $>$ 65 years, and in 10% of people aged $>$ 80 years.[1,2] In 2017, 12.6 million people were diagnosed with AS,[3] and the number of cases has increased over the years.[4] Patients diagnosed with severe AS are at high risk of heart failure and/or death within 5 years from diagnosis,[5] but no medical therapy is available. The only option is to replace the stenotic AV through surgical or transcatheter approaches.[6-9] Each year, more than 200,000 AV replacements and 20,000 deaths associated with AS are recorded in North America.[4,10] Thus, AS carries a significant economic and societal burden, and the need to develop alternative treatments to alleviate these burdens is urgent.

Many sex differences have been highlighted in AS, from clinical studies. AVs in female patients have more fibrosis and less calcification than do those in male patients, for the same hemodynamic parameters and the same severity of AS.[11,12] Differences also occur in the remodelling of the left ventricle, with more concentric hypertrophy in female patients, and more eccentric hypertrophy in male patients,[13] which leads to a sex-specific presentation of more low-flow with preserved ejection fraction in female patients.[14] These discoveries have been explained at the molecular level, but many mechanisms remain unexplained (Fig. 1). Several hypotheses have been proposed regarding major sex-specific factors in the development of AS, including lipid metabolism, immunity, hormones, and genetics. In this article, we aim to review the molecular mechanisms associated with AS, and we then focus on molecular differences between male and females, as highlighted in studies conducted mainly in experimental models.

Composition of the AV

Human AV leaflets are trilayered structures, each assuring different properties. The fibrosa, facing the aorta, is mainly composed of collagen fibers oriented circumferentially, which provides tensile stiffness.[15] The ventricularis, facing the left ventricle, is a layer of elastic fibers that gives the leaflets their compliance. Finally, the spongiosa, which separates the fibrosa from the ventricularis, is rich in proteoglycans and glycosaminoglycans, giving the leaflets elasticity and flexibility.[16] The AV is mainly composed of valvular endothelial cells (VECs), valvular interstitial cells (VICs), immune cells, and extracellular matrix (ECM), all of which play important roles in physiological and pathologic conditions (Fig. 2). All of these components may undergo and regulate calcification and fibrosis processes in a sex-specific manner.

Valvular endothelial cells

VECs form a protective monolayer[17] over the 2 faces of the AV.[15] In physiological conditions, these cells have multiple functions, such as secreting nitric oxide to regulate vascular homeostasis,[17,18] and replenishing the population of VICs.[17,19] VECs located on the ventricularis face experience shear stress from blood flow, whereas those on the fibrosa face are exposed to oscillatory shear stress. Under pathologic conditions, such as oscillatory shear stress, and inflammation, VECs may differentiate into osteoblasts via an endothelial–mesenchymal transition.[20,21] VECs also may inhibit the calcification of VICs by promoting the anti-osteogenic effect of NOTCH1.[22]

Sex differences in VECs are generally understudied. A recent study, on normal porcine AVs, observed that the proliferation of VECs differs between male and female individuals. Healthy male VECs exhibit a higher level of proliferation than do female VECs *in vitro*.[23] This difference might be due to higher levels of thrombospondin 2 being secreted by female VECs.[23]

Valvular interstitial cells

VICs are located throughout the AV leaflets, beneath the VEC layer. VICs have 4 subpopulations, as follows: progenitor; quiescent; activated; and osteogenic.[24] Progenitor VICs may originate from endothelial and hematopoietic lineages.[24-27] Progenitors from an endothelial origin may give rise to quiescent VICs, whereas those from a hematopoietic origin may give rise to activated VICs involved in valve repair.[24] Quiescent VICs (qVICs) maintain the balance between the synthesis and degradation of ECM components in the valve under physiological conditions.[21] In

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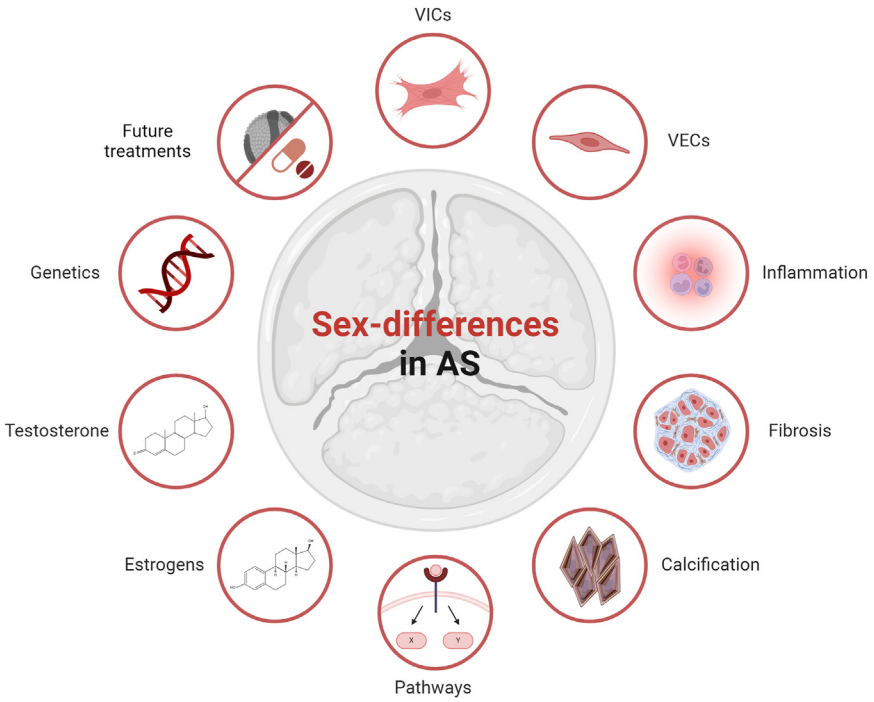

Figure 1. Sex-related differences observed in aortic stenosis (AS). VECs, valvular endothelial cells; VICs, valvular interstitial cells. Figure created with BioRender.com.

response to physiological stimuli, qVICs may get activated into myofibroblast cells, ensuring normal ECM remodelling and function.[21] This differentiation is thought to be reversible when stimuli recede.[21,28] Under pathologic conditions, qVICs and progenitor VICs (from both endothelial and hematopoietic origins) may transform into activated VICs (aVICs) capable of repairing the valve.[24] They may evolve into preosteoblasts or myofibroblasts promoting calcification.[25] This transition is associated with ECM remodelling and contributes to the fibro-calcific remodelling of the AV.[29] VICs might lose their pluripotency when AV calcification develops. *In vitro* experiments show that VICs extracted from normal valves can differentiate into myofibroblastic, osteoblastic, chondrogenic, or adipogenic lineages,[30] whereas VICs from calcified valves are less prone to differentiation into other cell types.[31]

Other studies provide complementary data that help in understanding the molecular mechanisms associated with sex differences in VICs. Male porcine VICs grown in osteogenic medium form bigger and more-dense nodules of calcium than do female VICs.[32] Also, male, compared to female, rat VICs express increased early osteogenic markers.[32] Female porcine VICs may have greater metabolic activity and collagen production than do male VICs,[33] but these later data seem controversial.[32] Altogether, these data suggest that stronger and earlier calcifying events occur in male VICs, and that more fibrotic events occur in female VICs. Thus, VICs may be important protagonists in sex-specific calcification and/or fibrosis processes.

An important finding is that VICs also may play a role in angiogenesis in a sex-specific manner. Female porcine VICs show a higher secretion level of vascular endothelial growth factor A (VEGF-A) than do male VICs, but only in qVICs. Basic fibroblast growth factor (also called FGF-2) production also tends to be higher in female porcine VICs.[23] However, VEGF-A is increased in AVs from male patients with severe AS, compared to that in female patients (Fig. 2).[34] Female porcine aVICs have more heparan sulfate proteoglycan-2,[23] a molecule that favours growth factor binding. All these studies show discrepancies between male and female VICs in angiogenesis, but further investigations are needed to clarify the conflicting results.

Other cell populations in the AV

A recent transcriptomic study differentiated 14 cell subtypes in stenotic AVs. Beside VIC and VEC subpopulations, 6 valve-derived stromal cell populations and 3 immune-derived cell populations were identified.[20] Macrophages, mast cells, and lymphocytes have been found to promote inflammation in stenotic AVs.[35,36] Smooth muscle cells represent a small proportion of AV cells and are found mostly in the ventricularis layer. In AS, smooth muscle cells are found near the calcified areas.[37]

Sex differences also may be present in these cell populations. A study from Myasoedova et al. recently showed that stenosed AVs from female individuals contain more mesenchymal cell signatures, whereas those from male individuals have more signatures from macrophages, monocytes, T cells, and B cells.[38] Another study conducted on human VICs shows that the osteogenic response induced by interferon-α and/or lipopolysaccharide is counteracted by the Ak strain transforming (Akt) pathway in female but not male VICs.[39] This result indicates that not only valve composition but also immune response may be sex specific in AS.

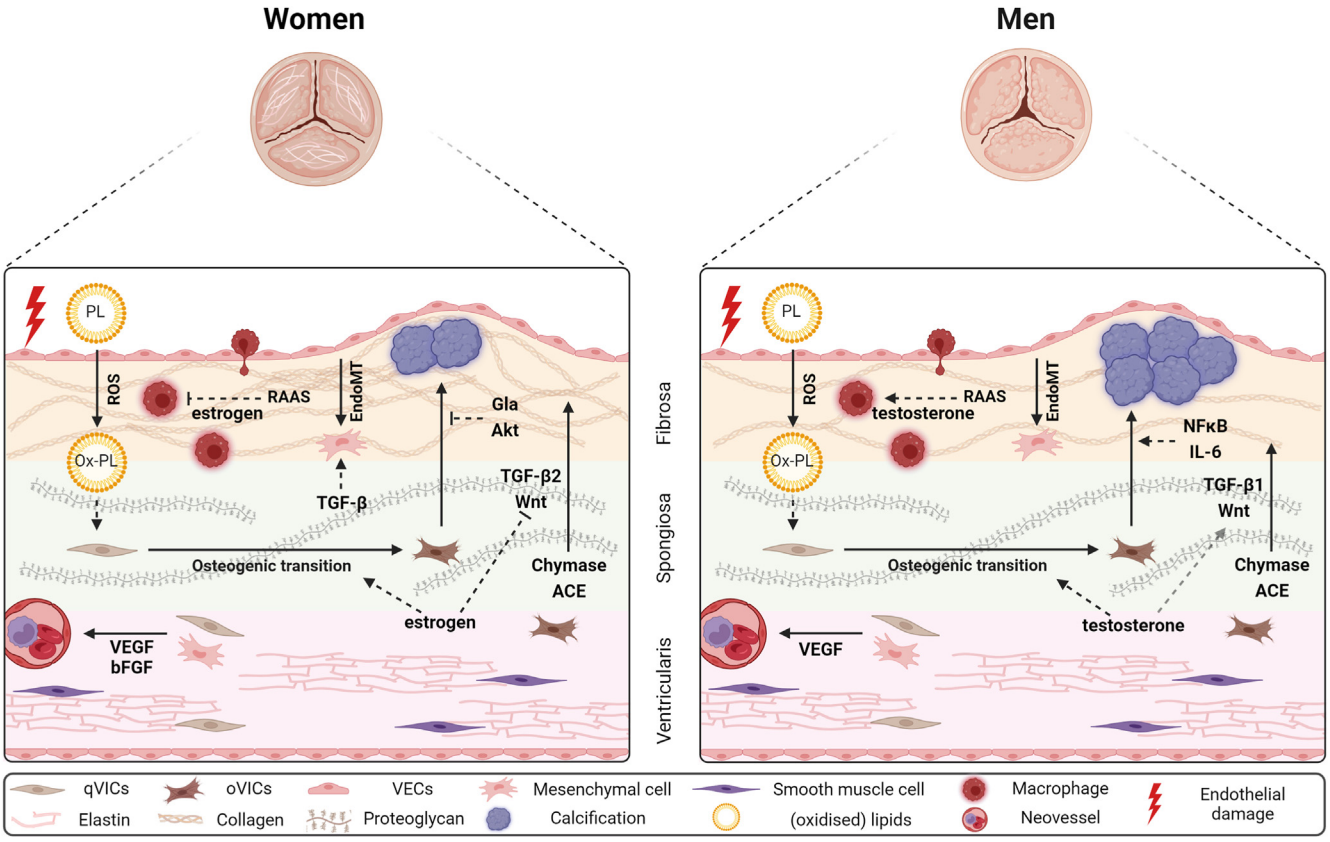

Figure 2. Pathophysiology of aortic stenosis (AS) and possible impact of sex hormones and sex differences. An endothelial lesion induces the infiltration of lipids, quickly followed by their oxidation, and inflammatory cells in the fibrosa. The renin-angiotensin-aldosterone system (RAAS) regulates valvular inflammation and seems to be anti-inflammatory in female patients through the effect of estrogen, and proinflammatory in male patients via testosterone. The transforming growth factor-β (TGF-β) pathway, upregulated in female patients, may promote the endothelial-mesenchymal transition (EndoMT) observed in AS. Valvular interstitial cells (VICs) are activated by several factors, including oxidized lipids. Their osteogenic transition seems to be inhibited by estrogen and promoted by testosterone, resulting in more calcification in stenotic aortic valves in male patients than in female patients. Moreover, the Ak strain transforming (Akt) pathway inhibits calcification in female patients, whereas the nuclear factor kappa beta (NFκB) pathway favours it through the receptor activator of nuclear factor κ B, receptor activator of nuclear factor kappa-B ligand, osteoprotegerin (RANK/RANKL/OPG) pathway in male patients. At the same time, chymase, produced by mast cells, and angiotensin-converting enzyme (ACE) induce fibrosis through the RAAS. Deposition of fibrosis is favoured by TGF-β1/β2 and wingless-related integration site (Wnt), a pathway upregulated by estrogen and potentially by testosterone. Vascular endothelial growth factor (VEGF) is an angiogenic factor secreted by male and female VICs in AS. In female patients, basic fibroblast growth factor (bFGF) also seems to promote angiogenesis. Gla, Matrix Gla protein ; ox, oxidized; PL, phospholipids; ROS, reactive oxygen species; VEC, valvular endothelial cells; VEGF, vascular endothelial growth factor; oVIC, osteoblastic VICs ; qVIC, quiescent VICs. Figure created with BioRender.com.

Extracellular matrix

The ECM differs in the 3 layers of aortic leaflets, as follows: the fibrosa is rich in collagen I and III; the spongiosa is rich in glycosaminoglycans; and the ventricularis is rich in elastin.[40] All these molecules confer specific mechanical properties to the AV.[41] In pathologic conditions, collagen fibers become degraded and disorganized, and they interact with calcified vesicles.[41] Several matrix metalloproteinases (MMPs), such as MMP1, MMP7, MMP9, and MMP12, are upregulated, whereas tissue inhibitor of metalloproteinase (TIMP) 4 is downregulated.[42]

The composition of the ECM is important in AS because its stiffness modifies the proliferation, differentiation, and viability of porcine VICs cultured in osteogenic media.[43] An interesting finding is that in cell culture, ECM composition is modulated by the sex of the VICs. In male individuals, the levels of glycosaminoglycans, collagen I, and activated MMP2 are higher than those in female individuals.[32] In female individuals, a higher level of expression and production of MMP2, MMP3, and MMP9, and a lower activity level of collagenase and gelatinase are observed, which is concordant with an accumulation of ECM.[33] It is important to note that 17β-estradiol inhibits the transcription of MMP2 through the mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 (MAPK-ERK1/2) signalling pathway in cardiac fibroblasts, suggesting that estrogens play a role in ECM remodelling.[44]

Pathophysiology of Calcified AS

The causes of AS are mainly unknown, but several mechanisms involved in the pathophysiology of the disease, including shear stress due to pressure overload, inflammation,

and oxidative stress, have been elucidated.[45] AS initiation appears when an endothelial lesion is observed.[46] This initiation is followed by lipid infiltration, mainly of low-density lipoproteins and lipoprotein (a), which triggers the expression of Toll-like receptors on VICs.[46] Reactive oxygen species are produced, oxidizing lipoproteins, which in turn induce the apoptosis of VICs and a cascade of immune reactions.[46] Many factors, such as the expression of cell adhesion molecule,[46] the recruitment of macrophages, CD11b,[47] and major histocompatibility complex (MHC) class II dendritic cells, are observed.[48] CD4+ and CD8+ T lymphocytes are attracted, mostly near areas of calcification or neovessels.[49] Mast cells secrete chymase, an enzyme promoting the production of angiotensin II and fibrosis in the AV.[50] Other mediators of inflammation, such as interleukin (IL)-6, cyclooxygenase 2, intracellular adhesion molecule 1 (ICAM1), and transforming growth factor beta-1 (TGF-β1), participate in the pathophysiology of AS (Fig. 2).[25,46,51]

Lipid infiltration, oxidative stress, and inflammation favour the differentiation of VICs, VECs, and macrophages into aVICs, a myofibroblast-like subpopulation of cells.[52] Myofibroblasts produce collagen,[53] a major constituent of fibrosis, and aggregate in apoptotic nodules; calcium deposits are produced around collagen fibers.[25] Concomitantly, VICs differentiate into osteoblasts responsible for the formation of true bone inclusions[25] and express markers of osteoblastic differentiation, such as Runt-related transcription factor 2 (RUNX2), bone morphogenic protein (BMP), alkaline phosphatase (ALP), and osteocalcin.[25,46] For unknown reasons, the pathophysiology of the disease shows a significant imbalance between male and female patients.[12] Male patients develop more calcifications, whereas female patients develop more fibrosis, for comparable hemodynamic parameters,[12] regardless of the phenotype of the AV.[54] An interesting point to note is that the immune response system may influence the fibrotic and calcific phenotypes observed, because immune cells such as monocytes, macrophages, T cells, and B cells are enriched in male individuals, whereas mesenchymal cells are enriched in female individuals.[38]

Signalling pathways

Various pathways are involved in AS. The renin-angiotensin-aldosterone system (RAAS) regulates blood pressure, and its chronic activation induces systemic hypertension, which has been linked to calcification in AS.[55] The receptor activator of nuclear factor κ B, receptor activator of nuclear factor kappa-B ligand, osteoprotegerin (RANK/RANKL/OPG) signalling pathway promotes the inflammatory response and calcification observed in AS. The Notch signalling pathway inhibits calcification, whereas the transforming growth factor-β (TGF-β) pathway favours the accumulation of fibrosis. The role of the wingless-related integration site (Wnt)/β-catenin pathway in AS needs deeper research, but it seems to regulate both fibrosis and calcification. These 5 pathways contribute to the development of AS and may be sex-specific (Fig. 3).

RAAS

Systolic hypertension is often concomitant with AS and is associated with faster progression of AV calcification.[56] Aldosterone and angiotensin are 2 molecules in this pathway that may be involved in AS in a sex-specific manner. In male VICs, aldosterone increases the expression of calcification markers, such as BMP2, BMP4, periostin, and osteopontin, through the mineralocorticoid receptor. However, in female VICs, aldosterone increases fibrosis markers (fibronectin, lumican, tissue inhibitor of metalloproteinase [TIMP]-1) via the same receptor.[57]

Under physiological conditions, angiotensin II regulates several parameters through its receptors AT1 and AT2.[58] Through AT1, it promotes vasoconstriction, fibrosis, inflammation, and oxidative stress, whereas through AT2, it promotes the opposite.[58] An interesting finding is that the AT1-receptor is the only receptor in the AV. Angiotensin II is produced in the AV via 2 enzymes—chymase and angiotensin-converting enzyme (ACE), both of which are overexpressed in stenotic AVs.[50,59] A recent study also showed that, in mice, treatment with angiotensin II increases the amount of collagen fibers (trend only).[60] The same work revealed that angiotensin II increases proliferation, activation, fibrosis, and TGF-β1 expression in VICs, but no information is given about the sex of the mice or the cells. Another study in mesangial cells suggests that angiotensin II may have a profibrotic effect by increasing the expression of ECM proteins through the induction of TGF-β.[61] At the physiological level, patients with AS are known to present high plasma levels of angiotensin II,[55] which is associated with high expression of inflammatory markers (IL-6 and tumour necrosis factor-α) in the AV.[62] These data suggest that RAAS may be involved in promoting valvular inflammation and fibrosis.

Knowledge is lacking regarding the sex-specific action of angiotensin II within the AV at the cellular level. Nevertheless, some data show that estrogen and testosterone may modulate the RAAS and thus modulate AS pathophysiology (Fig. 2). Estrogens promote high angiotensinogen levels (the precursor of angiotensin), low renin levels, ACE activity, aldosterone production, and angiotensin II type 1 receptor (AT1R) expression.[63] They downregulate the angiotensin I receptor in vascular smooth muscle cells.[64] In premenopausal female individuals, the RAAS seems to be cardioprotective via interactions with estrogens,[65,66] but intriguingly, an increase in the expression of angiotensinogen is observed in women treated with either estrogen-replacement therapy or contraceptive therapy.[67] Moreover, some studies show that RAAS may be influenced by hormonal changes during the menstrual cycle and in postmenopausal women.[68] In postmenopausal women, estrogen deficiency also deregulates the RAAS and leads to a proinflammatory state.[69]

In contrast, testosterone tends to increase renin levels and ACE activity.[70] In male individuals, testosterone can stimulate both vasodilator and vasoconstrictor pathways.[58,71] In contrast to estrogen, testosterone seems to amplify the pathologic features of the RAAS in cardiovascular diseases by favouring the angiotensin II-ACE-AT1 receptor pathway.[58] Estrogen and testosterone could partially explain the sex-related differences in the RAAS, but their effects seem to be tissue-specific and have not yet been studied in the AV (Fig. 3).

RANK/RANKL/OPG

This pathway is a well known major contributor to bone homeostasis. As calcification is an important feature of AS,

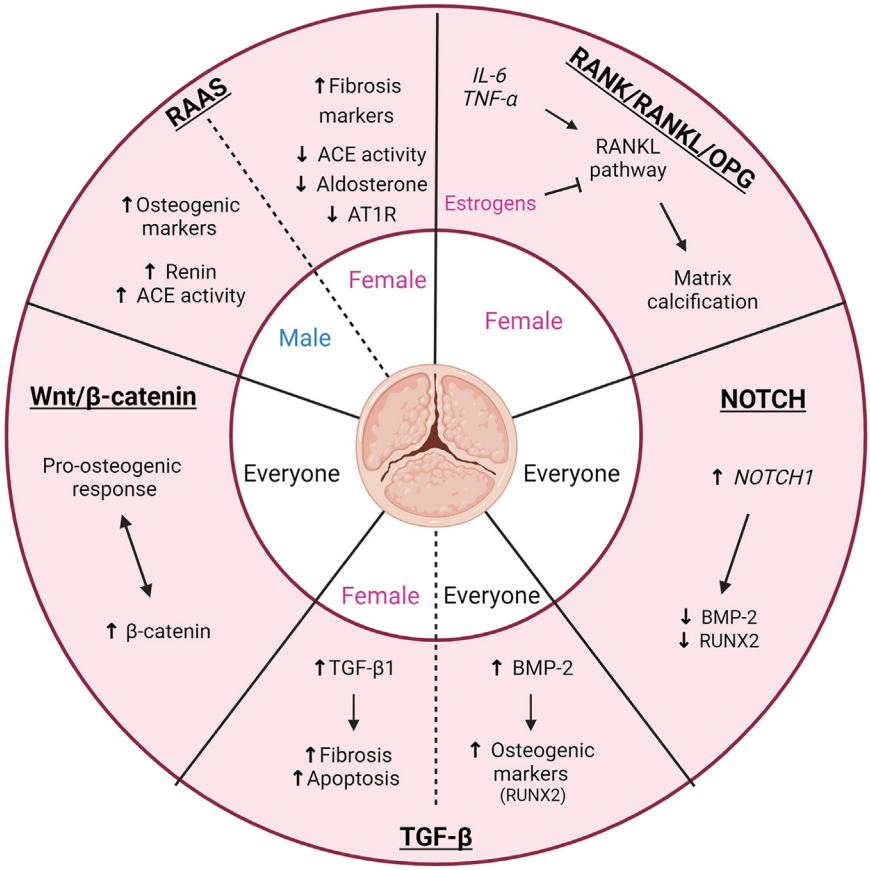

Figure 3. Sex differences in pathways involved in aortic stenosis (AS), and possible impact of sex hormones. Pathways leading to calcification and fibrosis may be modulated by sex hormones. In female patients, fibrosis markers may be increased through the renin-angiotensin-aldosterone system (RAAS), whereas in male patients, calcification markers may be upregulated. Estrogens may play a protective role in the calcification of the aortic valve by inhibiting the receptor activator of nuclear factor κ B (RANK) pathway. The Notch pathway may favour calcification processes and may be regulated by sex hormones, but no evidence has been found regarding its sex-specific regulation in AS. Several pieces of evidence show that transforming growth factor-β (TGF-β) may favour fibrosis in female patients. The wingless-related integration site (Wnt/β)-catenin pathway may favour a pro-osteogenic response in AS, but the involvement of sex hormones in its regulation needs to be clarified. ACE, angiotensin-converting enzyme; AT1R, angiotensin II type I receptor; BMP-2, bone morphogenic protein 2; IL-6, interleukine-6; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappa-B ligand; RUNX2, runt-related transcription factor 2; TNF-α, tumour necrosis factor alpha. Figure created with BioRender.com.

this pathway is of great interest. Studies in VICs show that the RANK/RANKL/OPG pathway is a downstream effector of tumour necrosis factor-α and IL-6, which promote calcification.[72-74] RANKL also promotes matrix calcification in human VICs and their osteogenic differentiation.[75] In vascular smooth muscle cells, RANKL is induced by oxidative stress and is under the control of RUNX2, a well known marker of calcification.[76]

RANKL is enhanced in the stenosed AVs of male, compared to female, individuals, suggesting that it has a role in the calcification of the AV in male individuals.[77] Even though female stenosed AVs have more fibrosis, they still exhibit some calcifications,[12,78] so the study of calcification in female AS is still of interest. In human aortic endothelial cells, and human aortic smooth muscle cells, estrogen interacts with estrogen receptor α and inhibits calcification through the RANKL pathway,[79,80] so the drop in estrogen in post-menopausal female individuals may increase RANKL activity and explain the limited calcifications observed in female patients with AS (Fig. 3). This modulation of the RANKL pathway may explain the cardioprotective role of estrogen in young female individuals, and the lack of calcification in young bicuspid female patients with AS.[54]

Notch

Notch is a transmembrane receptor protein involved in development, the differentiation of several cell types, and cardiac valvulogenesis.[81] *NOTCH1* mutations can cause developmental anomalies in the AV[82,83] and are correlated with serum levels of OPG and RANKL,[81] suggesting that Notch plays a role in valve calcification. Other studies suggest that Notch1 expression is decreased around calcification in AS, and that its downregulation in VICs increases calcification.[84,85] A surprising finding is that a high level of expression of its ligand, Notch Delta-like 1, has been associated with mortality in patients suffering from symptomatic AS and osteogenic differentiation in myoblastic and osteoblastic cell lines.[86,87] In VICs, increasing the expression of Notch1 decreases the expression of BMP2 and RUNX2 and prevents downstream mineralization (Fig. 3).[88] Experiments in human

endothelial kidney cells and osteoblastic lineages lead to the hypothesis that Notch1 repression may favour calcification in VECs and osteogenic differentiation in VICs.[85,89,90] In regard to sex differences, sex-specific modulation of the Notch pathway in fibroblasts occurs in response to hyperoxia, but more studies are needed to highlight these differences in AS.[91]

TGF-β

The TGF-β signalling pathway has 2 major roles in AS—it is both proapoptotic and profibrotic.[92-95] TGF-β1 attenuates calcification and osteogenic differentiation of VICs in a 3-dimensional model of AS,[96] and it favours cellular endothelial-mesenchymal transition.[97] Very interesting data show that TGF-β induces calcification in VICs if they are grown on uncoated tissue culture plates, but calcification is repressed when cells are grown on fibronectin surfaces.[98] These data mean that the TGF-β pathway in VICs may be sensitive to the microenvironment. BMPs belong to the TGF-β superfamily,[99] but they have opposing roles, as BMPs are osteogenic growth factors promoting calcification in AS. BMPs are upregulated in AS patients and in animal models.[100-102] For example, BMP-2 induces the expression of 2 pro-osteogenic factors, *RUNX2* and *SPP1*, in human VICs.[103] IL-6 promotes osteogenic differentiation of VICs and mineralization of the leaflets through the BMP-2 signalling pathway. [104] Like Notch1, BMP-2 is involved in embryonic cardiac valvular development.[105]

The balance between TGF-β and BMP signalling may differ in female and male AVs, and it could explain, at least in part, the sex-specific profibrotic and procalcific phenotypes observed.[106] Only a few studies have addressed this regulation according to sex, but we know that the TGF-β signalling pathway is upregulated in female, compared to male, patients with AS. Three genes in this pathway, *TGFβ2*, *MXRA5,* and *USP9X,* are overexpressed in female individuals[107]: they may contribute to the accumulation of fibrosis in female patients. This hypothesis is corroborated by the fact that TGF-β2 has a profibrotic effect through the Suppressor of Mothers Against Decapentaplegic pathway and high levels of ECM proteins.[108,109] TGF-β1 could have a different mechanism of action as it is overexpressed in calcifying conditions (male VICs and male AVs; Fig. 3).[33,34] Sex-related mechanisms associated with TGF-β mainly are unknown, but genes associated with the BMP and/or TGF-β have been shown to regulate X-chromosome inactivation in female individuals.[106,110]

Wnt/β-catenin

The Wnt/β-catenin signalling pathway is involved in developmental processes, through regulation of cell proliferation and bone homeostasis.[111] Low-density lipoprotein receptor-related protein 5 (LRP5), a co-receptor of Wnt, regulates the expression of bone matrix proteins in the AV and could modulate endochondral ossification in valvular heart diseases.[102,112,113] β-catenin is overexpressed in stenotic AVs and is correlated with the pro-osteogenic response induced by MMP12 in VICs.[114,115] The Wnt/β-catenin signalling pathway has been identified in AS, especially in its severe stages.[115,116] This pathway also induces fibrosis in human aortic VICs (Fig. 3).[117]

We demonstrated that 4 genes in the Wnt pathway (*DDX3X*, *GPC5*, *SFRP4*, and *LGR4*) are upregulated in female patients with AS.[107] An interesting finding is that estrogen receptor-1 modulates the expression of Wnt/β-catenin pathway genes, and estrogen receptor-α colocalizes with β-catenin in hepatocellular carcinoma.[118] In adipocyte lineages, testosterone regulates the Wnt/β-catenin signalling pathway, suggesting a possible sex regulation of Wnt/β-catenin in AS.[119] More studies are needed to determine how Wnt/β-catenin is regulated in female vs male individuals.

Sex hormones

Sex differences observed in AS may be associated with sex hormones. Circulating levels of testosterone have been correlated positively with vascular calcification, whereas serum levels of estrogen are associated negatively with vascular calcification.[80,120,121] Estrogen has a protective effect against cardiac fibroblast proliferation, and testosterone attenuates myofibroblast activation.[122,123] Androgen receptors and estrogen receptors type α and β are expressed in the AV, so they could play a key role in the sex differences found in AS.[124]

The underrepresentation of premenopausal female individuals in clinical studies slows the development of an understanding of estrogen's effects on the cardiovascular system.[80] Nevertheless, some studies show that estrogens exert their cardioprotective effect in premenopausal female individuals by modulating inflammation, lipoprotein metabolism, and vessel-wall properties.[125,126] In postmenopausal female individuals, a polymorphism in the estrogen receptor-α has been correlated with the development of AS.[127] Exposure to β-estradiol inhibits the proliferation of coronary smooth muscle cells in female, but not male, pigs.[128] The influence of β-estradiol on VIC behaviour needs further study, but it seems to inhibit the proliferation of VICs in female, but not male, rats.[32] An important finding is that 17β-estradiol inhibits the transcription of MMP2 through the mitogen-activated protein kinase extracellular signal-regulated kinase ½ (MAPK-ERK1/2) signalling pathway in cardiac fibroblasts, suggesting that estrogens play a role in ECM remodelling.[44] 17β-estradiol reduces fibrosis in female VICs only, but it does not affect calcification markers (BMP-4, RUNX2, osteopontin).[57]

The mechanism of action of androgens is poorly understood, but very interesting studies are emerging. First, Laukkanen et al. observed a correlation between high levels of testosterone and an increased risk of AS.[129] Then, Fleury et al. showed that testosterone increases procalcific genes and worsens hemodynamic parameters in a male mouse model of the disease,[130] but the direct mechanism of action of testosterone in the AV still is unknown. Testosterone may promote calcification of the AV via the androgen receptor, which is increased in stenotic AVs compared to normal AVs and is overexpressed in male, compared to female, individuals.[124] An important point to note is that testosterone induces calcification in vascular smooth muscle cells through the androgen receptor,[131] suggesting a similar mechanism of action in the AV, but the targeted cellular population still is unknown.

Genetic and Transcriptomic Studies Related to AS

Genetic factors are well known to be involved in the development of AS. Having a sibling with a history of AS

increases the risk of having AS by a factor of 3, whereas having a spouse diagnosed with AS increases the risk of developing AS only slightly.[132,133] Genetic factors seem to contribute more than environmental ones to the development of AS.[134,135] *LPA*, *PALMD, IL6, ALPL*, *NAV1,* and *TEX41* have been identified as susceptibility genes for AS, and single-nucleotide polymorphisms located near PALMD and IL1F9 are associated with AS.[133-139] AS pathophysiology differs according to the sex of patients, so determining genetic risk factors specific to sex is important. This determination has been done with Lp(a), whose single-nucleotide variants are associated with high serum levels of Lp(a) and a higher risk of AS in both male and female individuals.[140]

AS also has been characterized at the transcriptomic level. In 2009, Bossé et al. showed that 715 genes were differently expressed in normal vs stenotic human AV.[42] In healthy and stenotic valves, transcriptomic differences exist between male and female VICs and could explain the distinct VIC behaviour according to sex.[141,142] Indeed, profibrotic processes seem to be upregulated in female individuals, whereas proinflammatory pathways seem to be overrepresented in male individuals.[38] A recent study from our laboratory identified 190 genes that are expressed differently in female vs male patients with AS.[107] Genes correlated with fibrosis were overexpressed in female patients (*TGFβ2*, *KIF1A*, *FRAS1* . . .), whereas distinct genes linked to calcification were overexpressed in both female and male patients (female patients: *RCN2*; male patients: *CPAMD8* and *STC2*).

Epigenetics might also be involved in the sex differences observed in AS, because sex is known to influence the expression of microRNA.[143] Analysis has shown that 92 microRNAs are expressed differently in healthy vs stenotic AVs and may provide therapeutic targets for AS.[144] Another microRNA analysis revealed that thrombospondin 1 (*THBS1*) and nuclear factor kappa beta (NFκB) inhibitor α (*NFKBIA*) are shear stress—sensitive genes in AV.[145] However, this study does not describe male vs female differences.

Therapeutic Options and Biomarkers

Currently, no medical treatment is available to prevent or slow the progression of AS. When patients become symptomatic with severe AS, their prognosis is poor if they do not undergo surgical or transcatheter AV replacement.

The growing population with AS presents a critical need to develop medical treatments for AS, to reduce the burden of AV replacement and mortality. Several options have been tested, without success. Statins were proposed for AS treatment, owing to their efficacy in treating atherosclerosis. Several randomized controlled clinical trials using statins were conducted, but they did not slow the progression of AS.[6,7,146] Thus, statins are not recommended for AS treatment.

High levels of Lp(a) are associated with AS progression and may identify patients who need early AV replacement.[147] A therapy capable of reducing circulating Lp(a) levels may be promising for slowing AS progression. Pelacarsen (NCT05646381) and niacin (NCT02109614) currently are being tested in randomized controlled trials. Inclisiran has been shown to lower the low-density lipoprotein cholesterol level and the volume of Lp(a)-containing lipoprotein particles in atherosclerosis, so it would be interesting to test it in the context of AS.[148]

The RAAS is involved in fibrosis and AS progression, and it may be modulated in a sex-specific manner. In the **R**amipril **i**n **A**ortic **S**tenosis (RIAS) trial, an ACE inhibitor (Ramipril) led to a modest but progressive reduction of left ventricular mass in asymptomatic patients with moderate to severe AS, but it had no impact on AS progression.[149] This result could be explained by secondary production of angiotensin II by chymase in the AV.[50] The **A**ngiotensin **R**eceptor **B**locker in **A**ortic **S**tenosis (ARBAS) trial (NCT04913870) is currently underway to test the efficacy of angiotensin-receptor blockers in patients with mild-to-moderate AS. Whether the results differ for treated female vs male patients will be interesting to see; the study has been designed to provide sex-specific results.

As osteogenic pathways contribute to AS progression, especially in male patients, therapies targeting these pathways also have been tested. Denosumab and bisphosphonates are 2 pharmacologic agents targeting the procalcific nuclear factor kappa beta (NFκB)/RANK/RANKL/OPG pathway. The **S**cottish **A**ortic Stenosis and **L**ipid Lowering **T**rial: **I**mpact on **Re**gression (SALTIRE) 2 trial showed no impact on reducing AV calcification or slowing AS hemodynamic progression.[8]

Although AS diagnosis can be performed easily by mostly noninvasive methods such as echocardiography, many patients remain undiagnosed or are diagnosed late in the course of the disease, especially female patients.[11,12] Moreover, although the diagnosis of the disease is straightforward, evaluating its severity can be challenging,[14] and predicting the progression rate for a given patient is almost impossible. Therefore, blood biomarkers associated with the valve lesions that are capable of raising suspicion, improving diagnosis rates, or predicting progression rates would be of major interest in identifying patients at risk sooner, and tailoring the timing of follow up. As Lp(a) is associated with the occurrence of AS, it is used to identify patients at risk of AS, but it also could be used as a biomarker to raise suspicion for AS. Unfortunately, studies considering sex differences in blood biomarkers are lacking, in terms of both sex-specific impact and sex-specific thresholds.

The only available biomarkers in current clinical practice, in addition to Lp(a), are those associated with the impact of AS on the ventricle, such as N-terminal pro-B type natriuretic peptide (NT-proBNP) or high-sensitivity troponin T. These biomarkers are useful in conducting risk stratification and determining intervention timing.[150-154]

Conclusion

Female patients present a more profibrotic remodelling of their stenosed AV, whereas male patients present more calcification. The pathophysiology of the disease is a complex phenomenon, orchestrated by several pathways in which sex-related specificities are certainly involved, but major knowledge gaps remain. However, the volume of evidence of sex-specific regulation of pathways is currently increasing.

Aldosterone increases markers of calcification in both sexes and fibrotic markers in female patients only. The RANK/RANKL/OPG pathway, which promotes calcification, seems to be upregulated in male patients with AS, compared to female patients. Regarding fibrosis, the TGF-β signalling pathway is upregulated in female patients, compared to male patients, with AS. Sex hormones seem to influence the phenotype of the VICs, with estrogen possibly inhibiting

fibrosis, and testosterone promoting calcification and progression of AS. All these specificities may favour the fibrotic phenotype observed in female patients and the calcific phenotype observed in male patients with AS.

Aging is a risk factor for AS and a global characteristic of modern populations, so the need to develop medical therapies to slow or prevent AS is urgent. Given that the pathophysiology of AS differs according to sex, exploring sex-specific molecular mechanisms in AS is essential to developing sex-specific therapies.

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Ethics Statement

The authors confirm that review by ethics committee was not applicable to this article, as no patient nor animal data were used for this article.

Patient Consent

The authors confirm that patient consent is not applicable to this article, as no patient data were used for this article.

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